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TOWNSEND and TOWNSEND and CREW

By David Blankenship

PATENT

Attorney Docket No. 016994-003122

ORIGINAL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

H. DEBOER, et al.

Serial No.: 08/154,019

Filed: November 16, 1993

For: PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS

Examiner: J. Chambers

Art Unit: 1804

DECLARATION OF JUHANI JÄNNE UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Juhani Jänne, state as follows:

1. I am currently Professor of Biotechnology at the University of Kuopio (Finland) and hold several other academic positions including those of Director of the A.I. Virtanen Institute Univ. Kuopio and Vice-Chairman of the Department of Biochemistry and Biotechnology at the University of Kuopio. I am an editor and/or reviewer of over 30 scientific journals and am a coauthor of over 200 scientific articles. I have been awarded with several awards and honors for my research work including, most recently, the Kuopio prize of the city of Kuopio (in 1994) and the Matti Äyräpää prize of the Finnish Medical Society Duodecim (in 1994). A copy of my CV is attached.

2. I have been asked by Gene Pharming Europe to read their patent application PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS and to comment on the state of the art of transgenic cattle technology at the priority

date Of this application (December 1, 1989). I have further been asked to discuss the considerations that led to my own research efforts in this field. Therefore I have read and reviewed the above application as it was filed at the U.S. Patent and Trademark Office on December 1, 1989 [US 07/444,745]. I have further re-examined the background literature that is referenced in this declaration.

3. I have been active in the field of transgenic animals since I became a professor of biotechnology at the University of Kuopio in 1988. Before my move to Kuopio, my research interest was primarily focused on the metabolism and physiological function of polyamines. After I moved to Kuopio, I expanded that interest by also studying physiological of these molecules in transgenic mice and rats. I set up labs fitted to generating transgenic animals and have since generated many different lines of transgenic mice and rats. During the establishment of my research group in Kuopio, I decided that I should also form a group focused on production of valuable proteins (such as human erythropoietin) in milk of transgenic animals. I recruited my first Ph.D. student for this project in early 1990.

4. While contemplating the latter project and reading the relevant literature, it became clear to me that several people had succeeded in developing gene constructs that were capable of directing the expression of foreign proteins to milk of mice. Of course, I realized that mice were not well-suited as production animals as their milk yield is too low. On the other hand, I also realized that the methods used for generating transgenic mice were impractical to generate cows (who would be the ideal milk producers) with a reasonable efficiency. Briefly, transgenic mice are generated in the following way: females are superovulated by hormonal treatment after which they are allowed to mate. Several hours after mating, the female oocytes are removed from the oviducts of the females and are being injected with DNA. After injection, the oocytes are

implanted in the oviduct of a pseudopregnant female via a surgical procedure. It is unfeasible to use this procedure for the generation of transgenic cattle. An important reason herefore is that it would be prohibitively inefficient (and expensive!) to inject bovine oocytes obtained through superovulation as is done with mice. It would require large amounts of donor animals which would either have to undergo surgery or would have to be killed, in which case the animals cannot be used for human consumption anymore (due to the hormonal treatment). In addition, in contrast to mice, the number of oocytes per treated animal is low while it is also difficult and unpredictable to obtain oocytes from superovulated cattle that are in the right phase of the cell cycle for microinjection.

5. There had been reports in the literature that described attempts to generate transgenic cattle using, basically, the methods known for the generation of transgenic mice. For instance, Loskutoff et al. (*Theriogenology* (1986) 25:186) describe an effort to produce transgenic cattle. This abstract dramatically demonstrates the problems discussed in paragraph 4 of this declaration. A total of 75 surgical operations (51 to isolate oocytes and 24 to transfer the injected eggs) were needed to generate only three pregnancies. Given the fact that no follow-up paper was ever published, I assume that these pregnancies did not result in the birth of a transgenic calf. Biery et al. (*Theriogenology* (1988) 29:224) published an abstract in which they described microinjection experiments in bovine oocytes obtained through superovulation. Although this abstract lacks experimental detail, it is clear that large amounts of animals were used. Without showing scientific proof, it was reported that three transgenic fetuses were generated. However, in only one tissue sample (derived from placental material), transgene expression was, reportedly, detected. It is therefore likely that only mosaic integration of the transgene was achieved; mosaic integration means that the DNA is integrated after the one cell-stage of the embryo, resulting in animals in which only some tissues contain the transgene. It is further

relevant to note that there is no evidence in this abstract either that transgenic calves were actually generated. The above data, all based on oocytes obtained from superovulation (and fertilized *in vivo*), at the time indicated to me that alternative methods had to be developed in order to achieve a reasonable efficiency in the generation of transgenic cattle.

6. During the course of 1988 and 1989, several reports were published that described progress in culturing of bovine embryos *in vitro* (for instance, Eyestone and First (1989) *J. Reprod. Fert.* 85:715-720; Bondioli, PCT-patent application WO 89/07135; Gordon and Lu, European Patent Application 89303741.6). However, these references did not address the need to avoid methods to collect '*in vivo* oocytes' (using superovulation). Rather, they described methods to culture bovine embryos *in vitro* after fertilization had occurred. Although this was and is a relevant part of the entire protocol, I considered the source of oocytes as the real bottleneck of developing an efficient method of generating transgenic cattle.

7. Although protocols for *in vitro* maturation of bovine oocytes had been available for some time before I started contemplating a transgenic cattle project (in 1988 and 1989), it was my impression that the efficiencies that could be obtained were too low and too variable to be useful. For instance, Leibfield-Rutledge et al. (*Theriogenology* (1989) 31:61-74) stated in a review (summarizing the state of the art in *in vitro* maturation and fertilization) that *in vitro* oocyte maturation would need much improvement before it could be used for domestic animal production and research (which were the applications that these authors were contemplating). At that time, I believed that there was no reasonable expectation of success in using immature oocytes as a source for the generation of transgenic cattle. Certainly, the efficiencies as described by Gene Pharming Europe in their patent application, referred to in paragraph 2 of this declaration, were much higher than disclosed in the scientific literature at that time. It was not until 1990 that very high

efficiencies of *in vitro* maturation were reported in the literature.

8. Moreover, I realized that the data available at the time of the filing of the Gene Pharming application, or indeed, even after that filing date, did not address the effects that gene transfer protocols might have on the efficiencies of the entire *in vitro* process (i.e., the number of 'transgenic pregnancies' that could be reached by starting with a given number of immature oocytes). In fact, it was unpredictable how difficult or easy it would be to microinject *in vitro* matured oocytes as it could be expected that these cells would have different physiological properties compared to '*in vivo* eggs'; such an experiment had, to my knowledge, never been tried in any species. For instance, it was quite conceivable at the time that the phasing of the cell cycle of '*in vitro* oocytes' would be different from '*in vivo* oocytes'. This would have had an impact on the visibility of the pronuclei and, therefore, on the injection protocol. It was also quite conceivable at the time that the zona pollucida (the protein layer surrounding the oocyte) would have a different structure in '*in vitro* oocytes' compared to '*in vivo* oocytes'. Again, this would have impacted the injection protocol.

9. In early 1990 I became aware of a project being carried out by the University of Leiden in collaboration with researchers from Gene Pharming Europe and the Dutch Institute for Veterinary Research aimed at producing transgenic cattle by using immature oocytes as a source material. Apparently, these oocytes were matured and fertilized *in vitro*, with high efficiency, after which they were microinjected and further cultured *in vitro*. This integrated approach did increase the number of embryos available for transfer significantly. The overall efficiency of the process, although low in absolute terms, was unexpectedly high. It was certainly surprising at the time that this approach immediately resulted in transgenic pregnancies and transgenic calves born.

10. It is now clear to me that the use of *in vitro* methods has additional advantages. First, the use of *in vitro* methods drastically reduces the burden on individual animals as no surgical steps have to be performed. This is very important from an animal-welfare point of view, but also relevant from an economic point of view since the costs are significantly reduced. Second, the use of immature oocytes makes it possible to highly synchronize the maturation and fertilization. Therefore, injection of these oocytes will become more reproducible. Third, it should be noted that even in the most efficient superovulation regimens, a high proportion of non-transferable embryos resulting from fertilization failure and embryo degeneration reduce the usefulness of that method in cases when high numbers of fertilized embryos are needed. Fourth, the use of an *in vitro* method does allow for manipulation of the embryo (such as morphological examination, biopsy taking, etc.) at any stage of development.

11. Thus, within my department where expertise in molecular biology and transgenesis already existed, I formed a group of people building up expertise in the area of *in vitro* embryology and established a collaboration with an experimental animal research station. We started doing transfers of micro-injected (*in vitro*-cultured) bovine embryos in 1991. Our first transgenic calf was born in 1993. This work has been published recently (Hyttinen et al. (1994) *Bio/Technology* 12: 606-608).

12. As is apparent from the publication referenced in section 11 and from various other publications from my department we have been able to successfully follow the method such as described in [US 07/444,745] without incurring significant difficulties. Although the frequency of transgene integration is still quite low, the efficiency of the *in vitro* methods will ensure success by routine repetitions of the same procedures. I believe that the availability of an integrated process for *in vitro* generation of bovine embryos <u>starting from immature oocytes</u> has been critical for success. Not only did it allow for the

generation of transgenic bovine embryos, we have also taken advantage of the fact that the *in vitro* process makes it possible to perform embryo manipulations: we have taken biopsies from embryos and analyzed those before transferring the remainder of the embryos to recipient animals.

13. As is clear from our own work, combined with recent research efforts from other groups, the *in vitro* technology as discussed above (and described in US 07/444,745) is unrelated to the transgene employed. It is clear that it has general applicability to generate any type of transgenic bovine.

I have been duly warned that willful false statements and the like are punishable by fine and imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Juhani Jänne

November 23, 1995
Dated